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EXPERIMENTAL STUDY

Influence of Bushenhuoxue on podocytes of focal segmental glomerulosclerosis mice

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Abstract

OBJECTIVE: To observe the effects and mechanisms of Bushenhuoxue on desmin and nephrin expression in mice podocytes, and to investigate its effects on wt1 expression in Wilms' tumor.

METHODS: Adriamycin (ADR) was used to induce focal segmental glomerulous sclerosis (FSGS) in mice. Bushenhuoxue was used to treat FSGS for 6 weeks. We measured body mass and right renal mass, and determined serum albumin (ALB) levels, protein content in urine, and urinary protein and albumin creatinine ratio (UACR). Changes in renal tissue morphology were evaluated by microscopy. wt1 and nephrin expression in podocytes were detected using immunofluorescence. Expression levels of desmin, wt1 and nephrin mRNAs in renal tissue were determined using reverse transcription polymerase chain reaction assays.

RESULTS: Protein levels in urine and UACR were significantly increased in FSGS model mice compared with Bushenhuoxue-treated and control mice. Body mass and ALB levels were decreased in FSGS mice compared with control and Bushenhuoxue-treated mice. Expression of the wt1 protein was observed in control mice. Compared with controls, wt1 expression levels were reduced in Bushenhuoxue-treated mice, and to a greater extent in FSGS mice. Nephrin protein expression was widespread in FSGS mice, and significantly reduced in control and Bushenhuoxue mice. Expression levels of wt1 and nephrin mRNAs in FSGS mice were lower compared with those in control and Bushenhuoxue-treated mice. Desmin mRNA levels in FSGS mice were reduced compared with those in control and Bushenhuoxue-treated mice.

CONCLUSION: Bushenhuoxue ameliorated albuminuria in FSGS mice; this was possibly related to the up-regulation of wt1 and nephrin, and down-regulation of desmin.

Key word: Doxorubicin; Glomerulosclerosis, focal segmental; Podocytes; WT1 proteins; Nephrin; Desmin; Bushenhuoxue

INTRODUCTION

Focal segmental glomerulous sclerosis (FSGS) is a common, difficult to treat glomerular disease that can eventually lead to end-stage renal disease (ESRD). Its pathogenesis is not entirely clear, and treatment methods remain controversial.[1] Many studies have shown that podocyte injury at different stages is a key event of FSGS pathology.[2] Consequently, protecting injured

podocytes has become a key aspect of current FSGS treatments. The podocyte is an intrinsic, highly specialized kidney cell with limited regenerative ability. It is difficult for the podocyte to repair itself and proliferate when it is damaged or reduced. Podocyte mutations, and changes in their numbers and distribution can cause structural changes and induce albuminuria and glomerular sclerosis. Treatment of FSGS using western medicine does not result in a cure, it is expensive, and there are serious side effects. Traditional Chinese medicine could offer an effective way to treat FSGS.[3] Daningused Bushenhuoxue[4] to treat and cure renal diseases; Bushenhuoxue comprises greater than ten kinds of Chinese herbal medicines that promote blood circulation and *Qi*, remove blood stasis, and tonify the kidney. We focused on desmin, nephrin and wt1 expression in podocytes of FSGS mice, and their regulation by Bushenhuoxue. We also sought to elucidate the protective mechanisms of Bushenhuoxue on injured podocytes.

MATERIALS AND METHODS

Animals

We purchased 24 male BALB/c mice weighing 22-26 g, (6-8 weeks old) that were negative for albuminuria from the experimental animal center of the Academy of Military Medical Science(Certificate No. SCXK [jun] 2012-004). Mice were randomly divided into three groups according to random number tabal method: control (n=5); FSGS (n=10); and Bushenhuoxue (n=9). Our animal experiment was approved by the experimental animal ethics committee and we sought to reduce the suffering of animals as much as possible.

Reagents and drug preparations

Adriamycin (ADR) (St Louis, MO, USA) was mixed with physiological saline to a final concentration of 1 mg/mL. Bushenhuoxue was prepared by the Tianjin Institute of Pharmaceutical Research. Huangqi (*Radix Astragali Mongolici*),Wuweizi (*Fructus Schisandrae Chinensis*), Dahuang (*Radix Et Rhizoma Rhei Palmati*), Dahuang (*Radix Et Rhizoma Rhei Palmati*) (stir-frying to scorch), Yinchen(*Herba Artemisia Capillaris*), Danshen (*Radix Salviae Miltiorrhizae*),Chuanxiong (*Rhizoma Chuanxiong*),Wulingzhi (*Faeces Trogopteri*), Banzhilian (*Herba ScutellariaeBarbatae*), Jicai (*Herba Capsellae*), Shengma (*Rhizoma Cimicifugae Foetidae*). (Tai Ping Pharmacy, Tianjin, China) were concentrated into an extract with 90% (v/v) ethanol (Tianjin Baishi Chemical Co., Ltd. Tianjin, China) and preserved at - 20℃. For immunofluorescence, wt1 and nephrin antibodies were purchased fromSanta Cruz Biotechnology (Santa Cruz, CA, USA). A total RNA extraction kit, M-MLV reverse transcriptase, and an agarose gel electrophoresis kit was purchased from Takara (Tokyo, Japan). Thermal cyclers, a gel imaging system and a desktop high-speed centrifuge were purchased from Bio-Rad (Ontario, Canada). Primers were synthesized by Sango Biotech (Shanghai, China).[5]

FSGS animal model

After 1 week of adaptive feeding, mice in the FSGS and Bushenhuoxue groups were injected with ADR in the tail vein (10 mg/kg body weight). Mice in the control group were only once administeredsaline (10 mL/kg). We administered 10 μL of Boushenhuoxue via the intragastric route to each mouse in the Bushenhuoxue. Mice in the FSGS and control groups were then administered water (10 mL/kg). This treatment occurred every day for 6 weeks. By the end of the experimental period, there were six mice left in each of the FSGS and Bushenhuoxue groups.

Sample collection and processing

The body mass of each mouse was evaluated with an electronic balance before the end of the experiment. ALB, urinary albumin and urine creatinine concentration in collected blood and urine samples were assessed by high efficiency liquid chromatography. UACR was calculated using the following equation: UACR=urinary albumin (mg/L)×urine volume (mL)/[urinary creatinine (mM)×urine volume (mL)]. Mice were intraperitoneally injected with pentobarbital sodium anesthesia and the left kidney removed. The renal cortex was separated, with one third fixed in neutral buffered formalin for 24 h followed by dehydration, embedding, and slicing (3-μm thickness). Sections were stained with hematoxylin and eosin (HE) and periodic Acid Schiff-methenamine (PASM). Another third of the renal cortex was embedded in optimum cutting temperature (OCT) compound for use in immunofluorescence assays. The remaining left kidney was placed in liquid nitrogen and stored at - 80℃ for use in PCR assays. The right kidney was removed and weighed using an electronic balance, then placed in liquid nitrogen and stored at - 80℃.

Renal morphology

Sections were dewaxed and stained with HE and PASM. Morphological changes was observed using a light microscope.

Immunofluorescence staining

Renal tissue sections (3-μm thickness) were left at room temperature for 15 min after removing from - 80℃, then fixed with cold formaldehyde for 10 min followed by two washes with Tris-buffered saline with Tween (TBST). Sections were blocked with 2% bovine serum albumin (BSA) at room temperature for 1 h, then incubated with the appropriate primary antibody at 4℃ overnight. Sections were washed with Tris-buffered saline (TBS) three times (10 min each wash) and incubated with the appropriate secondary antibody at room temperature for 1 h in the dark. Nuclei were stained with DAPI and then washed three times with TBST (10 min each wash). Sections were mounted in

water-soluble mounting medium and observed using a fluorescence microscope.

Reverse transcription polymerase chain reaction (RT-PCR) assays

A Trizol kit was used to extract total RNA from kidney tissue. We used a UV spectrophotometer to assess the content and purity of each sample. Samples were also assessed by agarose gel electrophoresis. Areverse transcription kit was used to transcribe total RNA into cDNA. Reactions were incubated at 70℃ for 10 min, then placed on ice for 2 min, and incubated at 42℃ for 1 h after the addition of Rnase and M-MLV reverse transcriptase, incubated at 70℃ for a further 10 min, then placed on ice. Sequences of primers used in PCRs are listed in Table 1. Thermal cycling conditions for PCRs involved denaturation at 94℃ for 30 s, annealing at 60℃ for 30 s, and extension at 72℃ for 1 min, and total of 35 cycles. Glyceraldehyde 3-phosphae dehydrogenase (GAPDH) was used as a reference gene.

Statistical analysis

All values are presented as the mean ± standard deviation ($\bar{x} \pm s$). We used SPSS 17.0 (vers 17.0, SPSS Inc., Chicago, IL, USA) to analyze the data. One-way analysis of variance (ANOVA) was used to determine statistically significant differences among the groups followed by Fisher's least significant difference *t*-test. A *P*-value of 0.05 or less was considered significant.

RESULTS

General observations

Mice in the FSGS and Bushenhuoxue groups appeared to lose their appetite over the course of the experiment. Urine output was also decreased. Mice were apathetic with signs of edema, and had dull hair and lower activity levels.

Comparison of general data

Body mass and plasma ALB levels in Bushenhuoxue mice were significantly higher than those in FSGS mice ($P<0.01$). Urinary protein and UACR in Bushenhuoxue-treated mice were significantly increased compared with control group mice ($P<0.01$, Table 2).

Morphological changes in renal tissue

Staining with HE revealed that renal tissue morphology in the control group was normal. Proliferated mesangial matrix with fibrosis could be seen in the renal tissue from FSGS mice. Mesangial matrix with fibrosis in Bushenhuoxue-treated mice was significantly reduced compared with that in FSGS mice (Figure 1).

Staining with PASM showed that glomerular morphology and structure in control mice was normal. Glomerular basement membrane incrassation and segmental sclerosis was observed in FSGS mice and improved in Bushenhuoxue mice (Figure 2).

Immunofluorescence

We observed wt1 expression with normal distribution in the glomerular capillary of control group mice. wt1 expression was significantly down-regulated with segmental deletion in FSGS mice, and up-regulated in Bushenhuoxue-treated mice (Figure 3).

Nephrin expression in podocytes was distributed along the capillary in control group mice. For FSGS mice, nephrin expression was down-regulated and unevenly distributed. In the Bushenhuoxue-treated mice nephrin expression in the glomerular capillary was up-regulated compared with that seen in FSGS mice (Figure 4).

RT-PCR

Wt1 and nephrin mRNA expression levels in FSGS mice were lower compared with those in the control and Bushenhuoxue groups. Desmin mRNA expression

Table 1 Oligonucleotide primer sequences used in this study

Gene	Upstream primer sequence (5′-3′)	Downstream primer sequence (5′-3′)	Amplicon length (bp)
GAPDH	AAG AAC AGG CTC TTA GCA	CCA GTA GAC TCC ACG ACA T	134
Desmin	GCT TCG CCA ACT ACT TCG AG	GTG AGG TCT GGC TTG GAC AT	441
wt1	GGC ATC TGA GAC CAG TGA GAA	GAG AGT CAG ACT TGA AAG CAG T	400
Nephrin	CCA AGG TAC AGC CTG GAA GG	GAG ACA TCC TCT ACC GTG CG	311

Note: GAPDH: glyceraldehyde 3-phosphae dehydrogenase.

Table 2 Comparison of body mass, right renal weight, ALB level, urinary protein and UACR ($\bar{x} \pm s$)

Group	*n*	Body mass (g)	Right renal weight (g)	ALB (g/L)	Urinary protein (mg)	UACR (μg/moL)
Control	5	30.982±1.316	0.184±0.018	26.728±0.518	5.048±1.092	1.238±0.159
FSGS	6	24.640±4.306[a]	0.181±0.125	20.160±0.625[a]	30.547±1.531[a]	15.128±1.178[a]
Bushenhuoxue	6	28.917±0.668[b]	0.183±0.006	26.167±0.594[b]	13.976±1.548[b]	1.794±0.441[b]
F	-	6.306[c]	3.520	27.362[c]	195.163	626.976[c]

Notes: FSGS and control groups were administered water (10 mL/kg) once a day for 6 weeks. Boushenhuoxue group was administered 10 μL of Boushenhuoxue via the intragastric route once a day for 6 weeks. FSGS: focal segmental glomerulous sclerosis; ALB: albumin; UACR: urinary protein and albumin creatinine ratio. Compared with the control group, [a]$P<0.01$; compared with the FSGS group, [b]$P<0.01$; Statistically significant differences among the groups by One-way analysis of variance (ANOVA) determine, [c]$P<0.01$.

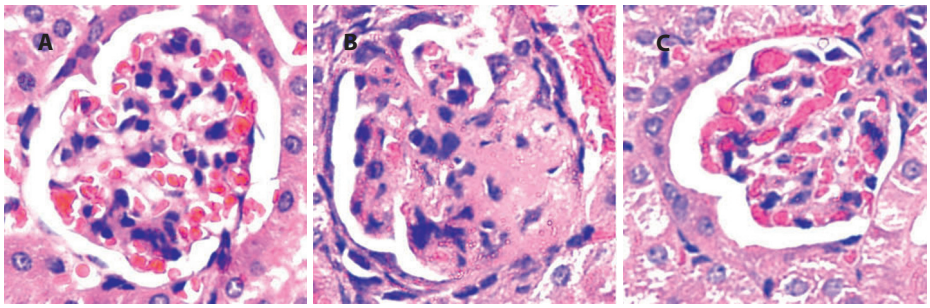

Figure 1 Photomicrographs of HE-stained sections among different groups (×400)
A: control group; B: FSGS group; C: Bushenhuoxue group. FSGS and control groups were administered water (10 mL/kg) once a day for 6 weeks. Boushenhuoxue group was administered 10 μL of Boushenhuoxue *via* the intragastric route once a day for 6 weeks. FSGS: focal segmental glomerulous sclerosis; HE: hematoxylin and eosin-stained.

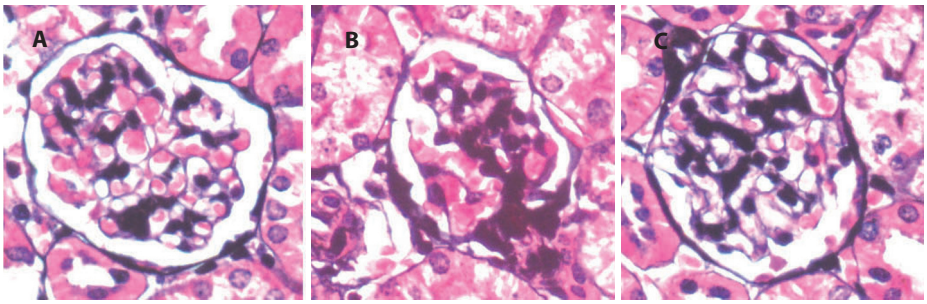

Figure 2 Photomicrographs of PASM-stained sections among different groups (×400)
A: control group; B: FSGS group; C: Bushenhuoxue group. FSGS and control groups were administered water (10 mL/kg) once a day for 6 weeks. Boushenhuoxue group was administered 10 μL of Boushenhuoxue *via* the intragastric route once a day for 6 weeks. FSGS: focal segmental glomerulous sclerosis; PASM: periodic acid silver methanamine-stained.

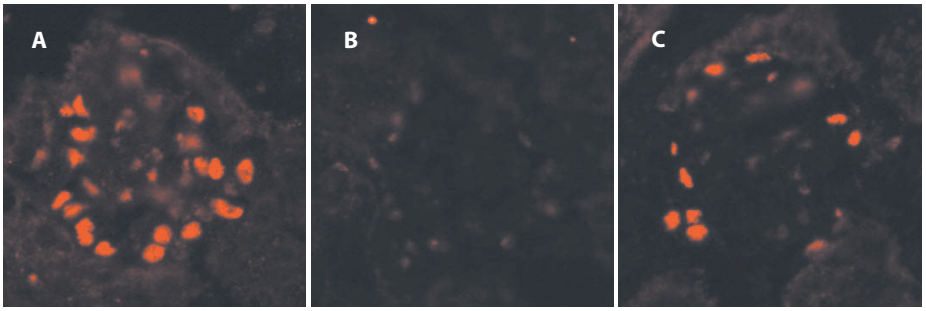

Figure 3 Immunofluorescence for wt1 expression changes expression among different groups (×200)
A: control group; B: FSGS group; C: Bushenhuoxue group. FSGS and control groups were administered water (10 mL/kg) once a day for 6 weeks. Boushenhuoxue group was administered 10 μL of Boushenhuoxue *via* the intragastric route once a day for 6 weeks. FSGS: focal segmental glomerulous sclerosis; wt1: wilms tumor 1 protein.

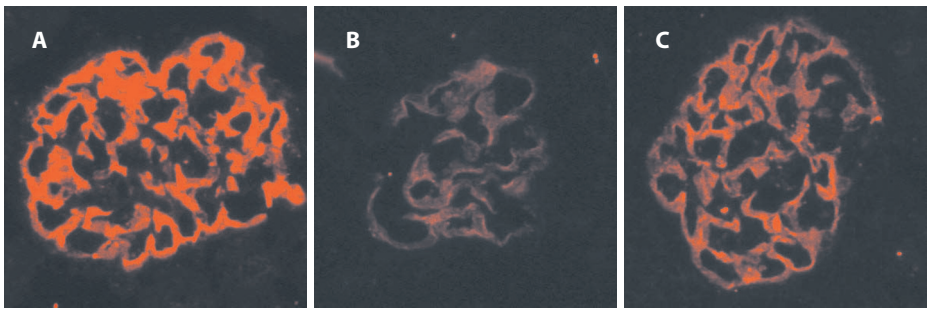

Figure 4 Immunofluorescence for nephrin expression changes expression among different groups (×200)
A: control group; B: FSGS group; C: Bushenhuoxue group. FSGS and control groups were administered water (10 mL/kg) once a day for 6 weeks. Boushenhuoxue group was administered 10 μL of Boushenhuoxue *via* the intragastric route once a day for 6 weeks. FSGS: focal segmental glomerulous sclerosis.

in FSGS mice was significantly increased compared with control group and Bushenhuoxue mice (P<0.01; Figure 5). Relative mRNA expression levels of desmin, wt1 and nephrin compared with that for GAPDH are presented in Table 3.

DISCUSSION

We used ADR to induce nephropathy in a classic animal model; nephropathy was characterized by massive proteinuria because of injury to podocytes. ADR dam-

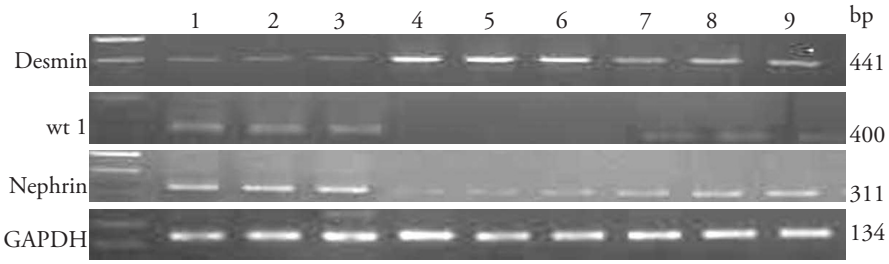

Figure 5 Changes in desmin, wt1 and nephrin mRNA expression levels

1-3: control group; 4-6: FSGS group; 7-9: Bushenhuoxue group. FSGS and control groups were administered water (10 mL/kg) once a day for 6 weeks. Boushenhuoxue group was administered 10 μL of Boushenhuoxue via the intragastric route once a day for 6 weeks. FSGS: focal segmental glomerulous sclerosis; wt1: wilms tumor 1 protein; GAPDH: glyceraldehyde 3-phosphae dehydrogenase.

Table 3 Comparison of desmin, wt1 and nephrin expression levels to GAPDH

Group	*n*	Desmin: GAPDH	wt1: GAPDH	Nephrin: GAPDH
Control (1)	5	0.115±0.014	1.328±0.041	1.497±0.310
FSGS (2)	6	1.896±0.102[a]	0.743±0.061[a]	0.204±0.143[a]
Bushenhuoxue (3)	6	0.892±0.319[b]	1.075±0.024[b]	1.157±0.418[b]
F	-	135.898[c]	209.247[c]	309.464[c]
P (1) : (2)	-	-	<0.005	<0.01
(2) : (3)	-	-	<0.005	<0.01
(1) : (3)	-	0.374	0.142	0.582

Notes: FSGS and control groups were administered water (10 mL/kg) once a day for 6 weeks. Boushenhuoxue group was administered 10 μL of Boushenhuoxue *via* the intragastric route once a day for 6 weeks. FSGS: focal segmental glomerulous sclerosis; wt1: wilms tumor 1 protein; GAPDH: glyceraldehyde 3-phosphae dehydrogenase. Compared with the FSGS group, [a]*P*<0.01; compared with control group, [b]*P*<0.008; statistically significant differences among the groups by One-way analysis of variance (ANOVA) determine, [c]*P*<0.01.

ages the membrane integrity of glomerular filtration and adversely affects normal physiological function, this results in proteinuria and formation of glomerular sclerosis, similar to that seen in human FSGS.[6]

Wt1 expression in FSGS model mice[7]

Wt1 is expressed at multiple stages during kidney development, but is restricted to podocytes following kidney formation and might indirectly reflect podocyte number. Ohtaka *et al* [8] showed that during FSGS, wt1 expression in injured podocytes was low. Our results indicated hyperplasia of the glomerular matrix and fibrosis in FSGS mice. We also showed that wt1 expression was down-regulated in FSGS mice by immunofluorescence and RT-PCR, suggesting a close relationship between wt1 deletion and FSGS.

Nephrin expression in FSGS model mice

Nephrin is mainly expressed in renal tissue. Patrakka *et al* [9] showed that slit membrane defects and macroalbuminuria could be observed in mice where nephrin was down-regulated. Nephrin expression was down-regulated in FSGS, with high levels of urinary protein but lower levels of nephrin expression.[10] Our results show that urinary albumin excretion and UACR in FSGS mice were increased, while ALB levels were decreased compared with those in control group mice. This likely contributes to decreased nephrin expression following podocyte injury, which leads to issues with the structural integrity of the glomerular slit diaphragm. A consequence of this is serious damage to the glomerular filtration barrier. We found that nephrin expression was decreased in FSGS model mice. The degree of hyperplasia in the glomerular matrix with fibrosis in FSGS mice was more serious than that in normal mice.

Desmin expression in FSGS model mice

The podocyte cytoskeleton can be rearranged and desmin expression induced after podocyte injury. Previous studies have shown that desmin expression is increased in FSGS patients. These results indicate that the degree of podocyte injury is positively correlated to urinary protein levels and negatively correlated to endogenous creatinine clearance rate. Podocyte injury plays an important role in the pathogenesis of proteinuria and renal hypofunction.[11] Our results showed that desmin mRNA expression levels and urinary protein levels in FSGS mice were increased compared with those in control mice, indicating that podocytes in FSGS mice were severely damaged. This can lead to destruction of glomerular filtration barrier integrity and large amounts of protein in the urine.

Bushenhuoxue treatment of FSGS

A Bushenhuoxue prescription provides a warming Yang and nourishing *Yin*, tonifies the kidney, activates blood, and promotes *Qi* to strengthen body resistance, cultivate the base, promote blood circulation and eliminate pathogens based on "kidney deficiency and blood stasis syndrome".[12-15] Previous studies have shown that

large doses of astragalus[16] and schisandra improve kidney damage and decrease urinary protein levels.[17] Oriental wormwood and scutellaria barbata have anti-inflammatory and antiviral effects,[18] and inhibit the generation of antigen-antibody complexes. Salvia, ligusticum wallichii, trogopterus dung and other medicines inhibit platelet aggregation, improve microcirculation, and have anticoagulation, anti-allergy and anti-inflammatory effects.[19-21] Bushenhuoxue can improve the environment, enhance immune function and assist with repairing injured renal tissue.[22]

We have demonstrated that Bushenhuoxue treatment of FSGS model mice for 6 weeks resulted in an improvement in their spirit, appetite and activity. This was also accompanied by an improvement in body mass, increase in ALB levels, and a decrease in UACR. Staining with HE and PASM demonstrated that fibrous depositions in FSGS mice treated with Bushenhuoxue were significantly reduced. Presence of wt1 and nephrin proteins in glomerular areas were increased. Expression levels of wt1 and nephrin mRNA in Bushenhuoxue-treated FSGS mice were up-regulated, while desmin mRNA were down-regulated in our PCR data. Our results suggest that Bushenhuoxue treatment of FSGS model mice regulates desmin, nephrin and wt1 expression in podocytes, protects injured podocytes, improves the integrity of podocyte structures, reduces urinary protein level and delays the development of FSGS.

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